

ASHI Lab Manual

Title: Pronase

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Section 2: Comparison of Technical Alternatives

The utilization of pronase-digested cells can be advantageous, as pronase cleaves the Fc receptor, reducing the opportunity for nonspecific binding of antibody to the Fc receptor. The use of pronase-digested lymphocytes can decrease the background in the assay and increase the sensitivity and specificity of the B cell flow cytometric crossmatch.

Additionally, pronase has been used for crossmatches when the patient is being treated with Rituximab, a humanized anti-CD20 monoclonal antibody. Due to the structural similarity between Fc receptors and CD20, pronase cleaves the CD20 from the surface of B lymphocytes, eliminating the target of Rituximab. As a consequence, CD19 and not CD20 must be used to delineate B cells in a population of pronase-digested cells.

It is important to note that not all laboratories and clinicians agree on the advantages of using pronase-digested cells in the flow cytometric crossmatch. Many laboratories have found that pronase-digested cells can be advantageous for increased sensitivity of the B cell crossmatch, but not for the T cell flow crossmatch since Fc receptors are only found on a small subset of T cells. In the T cell crossmatch, the background fluorescence can actually increase when using pronase-digested cells. Laboratories may feel that pronase digestion renders the lymphocytes too fragile for crossmatching. The use of frozen lymphocytes in a crossmatch may not be possible, as these cell preparations may be too fragile to withstand the stress of pronase digestion. It may also be challenging for laboratories to obtain enough cells for crossmatching following digestion. Additionally, some clinicians may feel that the pronase crossmatch is too sensitive for their clinical decision making process. Such limitations are important to note and discuss with clinicians if pronase-digested cells are to be used in the flow cytometric crossmatch.

It is at the discretion of individual laboratories as to the concentration of pronase used for digestion and the digestion time. Optimal pronase digestion will ensure adequate removal of Fc receptors and reduction in background while minimizing deleterious effects on cell integrity or expression of other surface molecules.

Section 3: Specimen Requirements

Fresh lymphocyte preparation (approximately 8×10^6 cells/ml) – cells can be collected from peripheral blood, lymph nodes or spleen. Cells should be resuspended in culture media without FBS prior to digestion. Performing pronase digestion on frozen cells can be problematic where the cells may be too fragile to withstand digestion, resulting in unacceptable cell loss. Each laboratory must decide if this practice is acceptable.

Section 4: Procedure

Pronase digestion removes Fc receptors from the cell surface and is performed on a lymphocyte preparation that has been purified for flow cytometry.

Required Reagents:

Reagent	Purpose
Pronase	Removal of Fc receptors and other surface molecules
dH ₂ O	Reconstitution of lyophilized pronase
DNase	Removal of DNA from lysed cells following pronase digestion
Phosphate Buffered Saline (PBS) or culture media	Diluent for stock solution of pronase
PBS or culture media with 5-10% fetal bovine serum (FBS)	Wash buffer following pronase digestion (also used to neutralize DNase, optional)
Polypropylene tubes	Glass or polystyrene tubes should not be used

Procedure:

1. Perform cell count on lymphocyte preparation to be digested.
2. Add cells to pronase solution at the appropriate pronase concentration (approximately 1 mg/ml) and lymphocyte concentration (approximately 8×10^6 cells/ml). It is important not to use any media with FBS prior to digestion as it can compete for pronase activity directed against the cells.
3. Incubate cells in 37°C incubator or water bath for 5–30 minutes. Digestion time varies from lab to lab. For example, some labs incubate for 15 minutes in a 37°C water bath and others incubate for 30 minutes in a 37°C heat block.
4. If desired, add DNase to remove residual DNA from lysed cells. Incubate at room temperature for approximately 30 seconds.
5. Wash digested cells 2-3 times with wash buffer. Resuspend cells in wash buffer.
6. Perform cell count, viability check and proceed with crossmatch.

Required Equipment:

N/A

Limitations of Assay:

1. A cell count must be performed before and after pronase digestion as the concentration will decrease after digestion. In cases where cells are very limited, pronase digestion may not be possible.
2. Cells should be used within a few hours of pronase digestion. After an extended incubation, it is possible for live lymphocytes to begin to regenerate the cleaved receptors on the cell surface.
3. Pronase digestion may render cells more fragile and lead to a decrease in the percentage of viable cells. Consequently many labs do not use pronase digestion on frozen cells.

Section 5: Interpretation/Reporting/Troubleshooting

The laboratory must establish a positive/negative cutoff channel shift for flow cytometric crossmatches with pronase-digested cells. The cutoff value may or may not be the same as for undigested cells. All ASHI standards applicable to flow cytometric crossmatch are applied to flow crossmatch with pronase-digested cells. Some laboratories use pronase crossmatches only, some use both regular and pronase crossmatches, and others use a combination of both (i.e. regular T-cell and pronase B-cell). It is necessary to have an established and validated positive/negative cutoff value for each type of crossmatch being used in your laboratory.

Section 6: Quality Control/Quality Assurance/Validation

Validation Essentials:

1. The concentration of pronase used for digestion and digestion time must be validated prior to clinical use. The concentration and digestion time must be high enough to yield appropriate removal of Fc receptors, but not too high as to damage the cells or cleave bystander molecules such as CD19 or HLA molecules. Control studies must be set up looking at a range of pronase concentrations and digestion times in order to determine what is optimal. Such studies may include evaluation of Fc or Fc-like receptor expression by flow cytometry and crossmatch of the digested cells with previously characterized sera. Additionally, the number of T and B lymphocytes before and after pronase digestion can be compared to ensure minimal cell loss with pronase digestion.
2. When pronase-digested cells are used for flow cytometric crossmatching, the laboratory must establish a positive/negative cutoff channel shift. The cutoff value may or may not be the same as for the crossmatch with undigested cells. All ASHI standards applicable to flow cytometric crossmatch are applied to flow crossmatch with pronase-digested cells.

Quality Control/Assurance:

1. Each new lot or batch of reconstituted pronase must pass quality control before use as the enzymatic activity of each lot/batch may vary. This is done by comparing the potency with the previous lot of approved pronase. Additionally, some laboratories compare the number of T and B lymphocytes with and without pronase digestion to ensure that the pronase does not cause unacceptable levels of cell destruction.

Safety Considerations:

1. Pronase is typically purchased as a powder and reconstituted with water. When working with pronase in its powdered form, use personal protective equipment. A respirator may be recommended to avoid breathing dust. Ensure adequate ventilation is available. Refer to the product Material Safety Data Sheet for specific safety guidelines prior to use.

Section 7: Clinical Considerations

The decision on whether or not to use pronase-digested cells depends on the clinicians and your laboratory. It is particularly useful for patients receiving Rituximab, but can be used for any patient. Crossmatches with pronase-digested cells can be useful in reducing the background fluorescence on the crossmatch and eliminating non-specific binding to the cells. However, some laboratories also believe that pronase digestion renders the cells too fragile and gives falsely elevated channel shifts.

Flow cytometric crossmatching is considered the gold standard of the lymphocyte crossmatch. The use of single antigen antibody identification systems by flow cytometry and Luminex now rival and possibly exceed the sensitivity of the flow cytometric crossmatch. It is important for laboratories to develop cutoff values that are clinically relevant across different systems and platforms. For example, if an antibody is considered positive by single antigen beads using Luminex, it should also be detectable by flow cytometric crossmatch. The sensitivity of crossmatching using pronase-digested cells must also be considered if the laboratory chooses to use this method.

Section 8: Challenges and Future Direction

Pronase-digested cells have been used for flow cytometric crossmatching in histocompatibility laboratories for over a decade. The flow cytometric crossmatch using pronase-digested and undigested cells remains a vital component of testing. While there is little or no movement toward another method of crossmatching, there is a desire to move toward virtual crossmatching. Virtual crossmatching involves predicting crossmatch compatibility based on unacceptable and/or acceptable donor HLA type. Virtual crossmatching has the potential of saving time in the allocation of deceased donor organs. It also could better facilitate the use of large scale living donor exchanges across the country.

The utility of virtual crossmatching depends on the accuracy of calling unacceptable and/or acceptable antigens for a patient. The cutoff used to determine unacceptable or acceptable must be concordant with the results that would be obtained using the flow cytometric crossmatch, with or without pronase-digested cells. In most cases, an actual flow cytometric crossmatch is performed even when a virtual crossmatch is used to determine organ allocation. This may be done retrospectively, but is typically still performed. Therefore, for a virtual crossmatch to be valid, the unacceptable and/or acceptable antigens called for a patient must agree with how these donor antigens will react with patient serum in a flow cytometric crossmatch. In cases where the sensitivity of a crossmatch with pronase-digested cells is greater than non-digested cells, the antibody identification schema must be more sensitive as well.